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# Flavonoids Released Naturally from Alfalfa Seeds Enhance Growth Rate of *Rhizobium meliloti*[1]

**Ueli A. Hartwig[2], Cecillia M. Joseph, and Donald A. Phillips***

*Department of Agronomy & Range Science, University of California, Davis, California 95616*

## ABSTRACT

**Alfalfa (*Medicago sativa* L.) releases different flavonoids from seeds and roots. Imbibing seeds discharge 3′,4′,5,7-substituted flavonoids; roots exude 5-deoxy molecules. Many, but not all, of these flavonoids induce nodulation (*nod*) genes in *Rhizobium meliloti*. The dominant flavonoid released from alfalfa seeds is identified here as quercetin-3-*O*-galactoside, a molecule that does not induce *nod* genes. Low concentrations (1–10 micromolar) of this compound, as well as luteolin-7-*O*-glucoside, another major flavonoid released from germinating seeds, and the aglycones, quercetin and luteolin, increase growth rate of *R. meliloti* in a defined minimal medium. Tests show that the 5,7-dihydroxyl substitution pattern on those molecules was primarily responsible for the growth effect, thus explaining how 5-deoxy flavonoids in root exudates fail to enhance growth of *R. meliloti*. Luteolin increases growth by a mechanism separate from its capacity to induce rhizobial *nod* genes, because it still enhanced growth rate of *R. meliloti* lacking functional copies of the three known *nodD* genes. Quercetin and luteolin also increased growth rate of *Pseudomonas putida*. They had no effect on growth rate of *Bacillus subtilis* or *Agrobacterium tumefaciens*, but they slowed growth of two fungal pathogens of alfalfa. These results suggest that alfalfa can create ecochemical zones for controlling soil microbes by releasing structurally different flavonoids from seeds and roots.**

Alfalfa (*Medicago sativa* L.) produces flavonoid signals that induce nodulation (*nod*) genes in *Rhizobium meliloti* bacteria required in the formation of $N_2$-fixing root nodules (10, 17, 21). Seeds release luteolin (3′,4′,5,7-tetrahydroxyflavone) and chrysoeriol (3′-methoxyluteolin), as well as other 3′,4′,5,7-substituted flavones that are inactive *nod* gene inducers (10). In contrast, young seedling roots exude the 5-deoxy *nod*-gene-inducing flavonoids (flavone numbering system, Fig. 1) 4,4′-dihydroxy-2′-methoxychalcone, 4′,7-dihydroxyflavone, and 4′,7-dihydroxyflavanone (17). An additional flavonoid, formononetin (7-hydroxy-4′-methoxyisoflavone), also is released from seedling roots under stress (18). Genetic and biochemical evidence indicates that flavonoids induce rhizobial *nod* genes by activating a regulatory *nodD* gene product (15). A broader role for flavonoids in rhizosphere biology is suggested by the fact that luteolin also induces chemotaxis in *R. meliloti* through a *nodD*-dependent process (4).

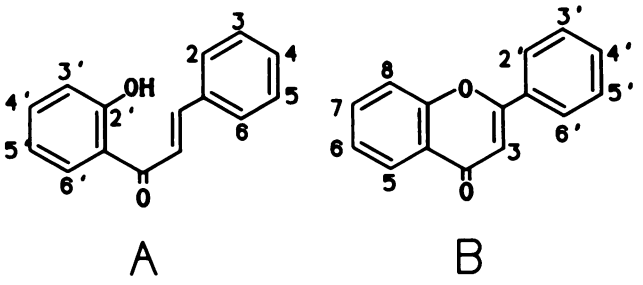


**Figure 1.** Carbon numbering conventions for flavonoids in this study. A, Chalcones; B, flavones, flavanones, flavonols (3-hydroxyflavones).

While alfalfa seeds release numerous 3′,4′,5,7-substituted flavones, the active *nod* gene inducers luteolin and chrysoeriol are minor components. The dominant flavonoid reported in alfalfa seed rinses was not identified because it was inactive in *nod*-gene-inducing assays (10). Another major flavonoid in that fraction was identified as luteolin-7-*O*-glucoside, a possible storage form of *nod*-gene-inducing activity, which was active only at concentrations 40-fold higher than free luteolin. Whether luteolin-7-*O*-glucoside and/or other major flavonoids lacking significant *nod*-gene-inducing activity have additional biological roles in the rhizosphere is unknown.

The purpose of the present study was to explore the biological implications of structural differences between flavonoids released from alfalfa seeds and roots. One objective was to determine how the dominant flavonoid in seed rinse differed structurally from *nod*-gene inducers in that fraction and to establish whether it too had 3′,4′,5,7-substitutents in contrast to the root flavonoids. When initial experiments showed that micromolar concentrations of seed flavonoids increased growth rate of *R. meliloti*, that potentially important mechanism for plant control of soil microbes was explored more fully.

## MATERIALS AND METHODS

### Culture and Treatment of Organisms

'Moapa 69' alfalfa (*Medicago sativa*) seeds were imbibed and rinsed with water to collect seed exudate as described previously (10).

Microorganisms were obtained from the following sources: *Rhizobium meliloti* 1021 (19), S. R. Long, Stanford Univer-

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[2] Present address: Institute of Plant Sciences, Federal Institute of Technology, 8092 Zurich, Switzerland.

sity; *R. meliloti* A2012 (1021 *nodD*$_1$::Tn5(9B7) *nodD*$_2$::tm *nodD*$_3$::sp/g-1) (12), F. M. Ausubel, Harvard Medical School; *R. meliloti* 445 (23), W. R. Ellis, USDA/University of Minnesota; *R. meliloti* 102F28 and *R. trifolii* 102X99, Nitragen Co., Milwaukee, WI; *Agrobacterium tumefaciens* C-58 (27), L. Smith, UC Davis; *Bacillus subtilis* ATCC 6051, *Pseudomonas putida* ATCC 12633, and *Pythium irregulare* (9) and *P. ultimum* (8), J. G. Hancock, UC Berkeley.

Bacteria were maintained on a complex medium and shifted to a defined minimal medium for experiments. Both media were adjusted to pH 7.0 and contained the following constituents, mg·L$^{-1}$: $KH_2PO_4$, 300; $Na_2HPO_4$, 300; $MgSO_4 \cdot 7\ H_2O$, 100; $CaCl_2 \cdot 2\ H_2O$, 5; $H_3BO_3$, 10; $ZnSO_4 \cdot 7\ H_2O$, 1; $CuSO_4 \cdot 5\ H_2O$, 0.5; $MnCl_2 \cdot 4\ H_2O$, 0.5; $Na_2MoO_4 \cdot 2\ H_2O$, 0.1; $FeCl_3 \cdot 6\ H_2O$, 0.1; thiamine·HCl, 1; biotin, 1; and Ca pantothenate, 1. The maintenance medium was supplemented with 2.5 g·L$^{-1}$ yeast extract and 15 g·L$^{-1}$ agar. The minimal liquid medium contained 2.5 g·L$^{-1}$ $NH_4NO_3$ without yeast extract. The carbon source for rhizobia was 1.0 g·L$^{-1}$ mannitol, unless stated otherwise; for *A. tumefaciens, B. subtilis,* and *P. putida,* 1.0 g·L$^{-1}$ glucose was used. *Pythium* was maintained on ATCC medium 343 (13) with agar, 15 g·L$^{-1}$. Several experiments with *R. meliloti* 1021 were done in a complex liquid medium (TY) containing tryptone (5 g·L$^{-1}$), yeast extract (3 g·L$^{-1}$), and $CaCl_2 \cdot 2\ H_2O$ (0.87 g·L$^{-1}$) at pH 6.8.

Bacterial growth in liquid media was monitored with a Lambda 6 UV/visible spectrophotometer (Perkin Elmer, Norwalk, CT) by absorbance at 600 nm ($A_{600}$). Viable cell counts were determined after dilution in sterile deionized water by plating on the yeast-extract agar medium. Protein content was measured with a Bio-Rad protein assay kit (Richmond, CA) after treating cells for 30 min with 5 N NaOH and neutralizing with HCl.

### Sources and Preparation of Flavonoids

Flavonoids were obtained from the following sources: luteolin, luteolin-7-*O*-glucoside, quercetin, quercetin-3-*O*-galactoside, formononetin, 3′,4′,7-trihydroxyflavone, 4′,7-dihydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, and 5,7-dihydroxyflavone (chrysin) (Spectrum Chemical, Gardena, CA); flavone (Sigma Chemical, St. Louis, MO); 4′,5,7-trihydroxyflavone (apigenin) (Aldrich Chemical, Milwaukee, WI); and 4,4′-dihydroxy-2′-methoxychalcone (R. Carlson, Ecochem Research, Chaska, MN).

Flavonoids normally were prepared gravimetrically as 2.5 or 5.0 mM stock solutions in methanol with warming. When cultures were supplemented with luteolin-7-*O*-glucoside, that flavonoid was supplied from a 100 μM solution prepared in minimal medium. In some experiments, flavonoid concentrations in liquid media were measured by HPLC analysis using an integrator function in the Waters 990 software package and authentic standards prepared with appropriate extinction coefficients (10, 17, 18).

### Flavonoid Identification

Flavonoids in 'Moapa 69' alfalfa seed exudate released during the first 4 h of imbibition were purified on a Waters HPLC system (Millipore, Milford, MA) equipped with a 250 × 10 mm Lichrosorb 5RP18 semipreparative reverse-phase column (Alltech, Deerfield, IL) using methanol:water:acetic acid gradients and a Waters 990 photodiode array detector as described previously (10). UV/visible spectral shift analyses (16) were done with the Lambda 6 spectrophotometer. One-dimensional proton NMR experiments were done in [U-$^2$H] acetone (Sigma Chemical, St. Louis, MO) on a Nicolet NT-360 spectrometer. Sugar conjugates were determined after acid hydrolysis (2).

### Growth Experiments

Growth tests of *R. meliloti* were done with cultures produced by two methods: In one case, cells were subcultured from a yeast extract agar slant to the minimal liquid medium, were monitored for growth at $A_{600}$, and, when the $A_{600}$ value doubled, were transferred to new minimal media containing flavonoids. This procedure produced *R. meliloti* 1021 cells with a doubling time ($g$)[3] of 11 to 15 h for cultures lacking flavonoids. The second method used cells that had been subcultured repeatedly and maintained on the minimal medium before flavonoid experiments were initiated. *R. meliloti* 1021 cells subjected to this procedure had $g$ values of 40 to 90 h without flavonoids. In all experiments with *R. trifolii, A. tumefaciens, B. subtilis,* and *P. putida,* cells were studied after being maintained in the minimal medium by the second method.

Bacterial growth experiments were conducted with 20 mL of medium in 125-mL Erlenmeyer flasks shaking vigorously in a 28°C waterbath. Flavonoids were added to the medium by diluting no more than 80 μL of methanol stock solutions into 20 mL of sterile minimal medium at 60°C. Open flasks then were cooled for 30 min in a laminar flow hood. Control flasks were supplied with 80 μL of methanol lacking flavonoids. Cells in early logarithmic growth phase were inoculated into the medium to give $A_{600}$ values near 0.040, which corresponded to about $10^7$ viable cells·mL$^{-1}$ for *R. meliloti* 1021. Optical density measurements were made every 2 to 6 h, depending on the doubling time of the culture. For each flask, 7 to 11 data points that indicated unrestricted growth (20) were analyzed for treatment effects on the specific growth rate constant ($k$) ($\log_{10} A_{600} = kt/2.303 + C$; $t$ = culture age, $h$; $C = \log_{10} A_{600}$ at $t = 0$) using statistical tests of linear regressions in SigmaPlot 4.0 (Jandel Scientific, Corte Madera, CA). The $g$ value was calculated from $k$ according to the relationship $g = (\ln 2)/k$ (20). Every experiment was conducted at least twice with two replicate flasks. When flavonoid treatments gave positive effects on growth rate, results were verified in at least three experiments with three replicate flasks.

Growth studies of the *Pythium* species were initiated by taking 5-mm plugs of mycelium equidistant from the edge of a culture growing rapidly on the standard agar medium and transferring them to 8-cm Petri dishes containing a 10-fold dilution of the maintenance medium. Flavonoids were supplied by placing crystals of each compound (1 mg·100 mL$^{-1}$) into sterile, 60°C medium and stirring for 10 min. Plates were incubated at 23°C and monitored for growth by measuring

[3] Abbreviations: $g$, doubling time; cfu, colony forming units.

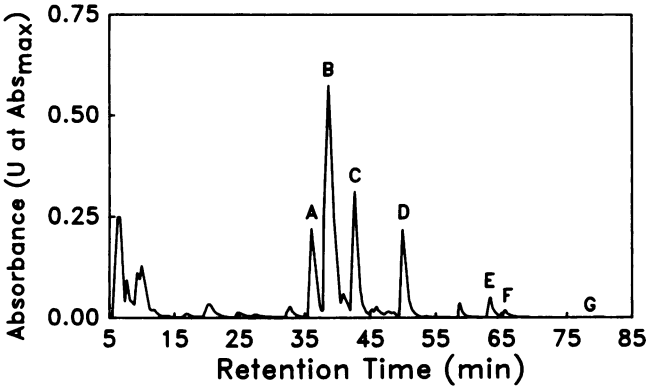


**Figure 2.** HPLC chromatogram of flavonoids exuded by alfalfa seeds during a 4-h aqueous rinse. Peaks indicated with letters have been identified as follows: A, luteolin-7-*O*-glucoside (10); B, quercetin-3-*O*-galactoside (this study); C, quercetin conjugate (this study); D, 5-methoxyluteolin (10); E, 3′,5-dimethoxyluteolin (10); F, luteolin (10); and G, 3′-methoxyluteolin (chrysoeriol) (10). Quercetin aglycone, which was not detected in this seed exudate sample, has a retention time of 63 min in the methanol:water:acetic acid gradient used.

the diameter of the mycelium every 12 h. Every experiment was conducted twice with five replicate plates.

## RESULTS

### Identification of Quercetin-3-*O*-Galactoside

Hydrolysis of peak B (Fig. 2) produced a galactose residue in GC analyses. NMR experiments and UV/visible spectral shift tests of the unhydrolyzed sample yielded data consistent with published values for quercetin galactosides (16) and sustained a tentative identification as quercetin-3-*O*-galactoside . (3′,4′,5,7-tetrahydroxyflavone-3-*O*-galactoside). This conclusion was supported by UV/visible spectral shift tests, HPLC cochromatography, and NMR analyses with an authentic standard. Supporting data, which were supplied to the reviewers, are available from the authors.

Peak C (Fig. 2) probably also is a quercetin conjugate. Acid hydrolysis of both peaks B and C produced a compound that cochromatographed with quercetin and gave UV/visible spectra identical to that compound.

These results and identifications made previously for compounds in the flavonoid fraction of alfalfa seed exudate (10) indicate that, during the first 4 h of imbibition, approximately 46% of that fraction consists of quercetin or its derivatives, while 22% of the fraction is luteolin or its derivatives (Fig. 2). Peak B, identified here as quercetin-3-*O*-galactoside, is the dominant flavonoid and constituted 32% of the flavonoid fraction in the seed exudate. Such estimates assume that unidentified flavonoids in the seed exudate are not quercetin or luteolin conjugates, an assumption consistent with UV/visible spectral data.

### Biological Activity of Flavonoids

Initial tests of whether *Rhizobium meliloti* 1021 metabolizes luteolin-7-*O*-glucoside or quercetin-3-*O*-galactoside (Fig. 2, peaks A and B) suggested the compounds had major effects on slowly growing bacterial cells. When luteolin-7-*O*-glucoside, for example, was supplied to cells at two stages of growth on a defined minimal medium, a more pronounced increase in cell density occurred in slowly growing cells (Fig. 3).

A growth effect similar to luteolin-7-*O*-glucoside was elicited with luteolin aglycone at concentrations as low as 0.25 μM (Fig. 4). A 5-μM treatment in the experiment reported in Figure 4 did not increase growth rate above that for 2.5 μM, and other tests with saturating concentrations of luteolin (20–30 μM in this medium, as determined by HPLC analysis) never increased the growth rate over the enhancement evident with 1 to 5 μM treatments in various experiments. Growth rates calculated for data in Figure 4 indicated that $g$ decreased from 11.8 h for the control cells to 5.9 h for cells treated with 2.5 μM luteolin. Adding luteolin to the yeast extract-tryptone medium did not alter the normal 3-h $g$ value of cells under those conditions.

Tests with other flavonoids identified in alfalfa seed and root exudates indicated that only luteolin, quercetin, and their glycosides increased growth rate of *R. meliloti* 1021 (Table I). No compounds tested from root exudates decreased the doubling time, but there was a significant slowing of growth with those flavonoids. In this experiment, flavonoid concentrations in the medium were measured by HPLC before inoculation with bacteria. This precaution was taken because flavonoid aglycones are not easily soluble in water. Flavone concentrations estimated gravimetrically from methanol dilutions into aqueous media frequently were larger than the value measured by HPLC (*e.g.* a projected 10 μM concentration often yielded 5 μM). Data in Table I are typical of several experiments started with bacteria that had doubled once on the minimal

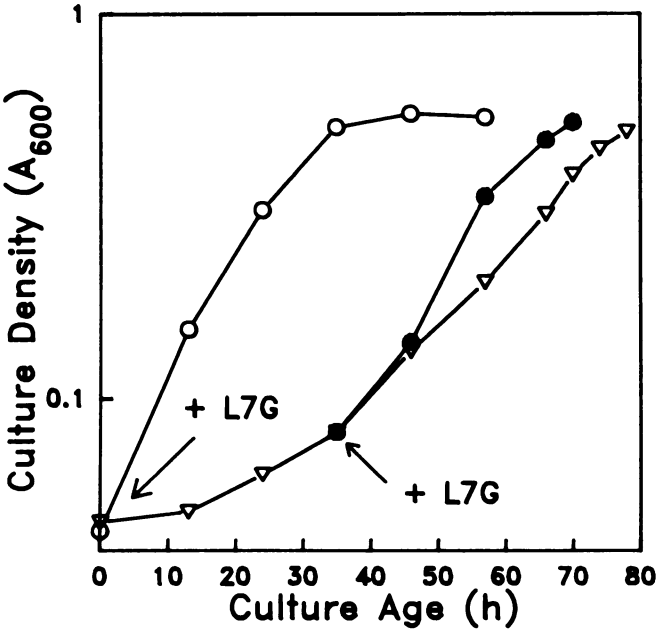


**Figure 3.** Effect on growth of *R. meliloti* 1021 caused by supplementing the medium to contain 10 μM luteolin-7-*O*-glucoside. The glucoside was added to control cultures (▽) at 0 (○) or 37 h (●) after the experiment started.

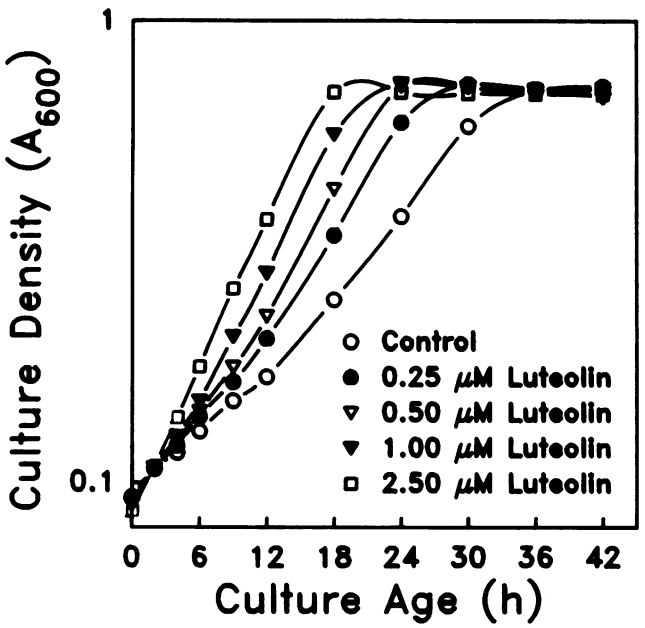


**Figure 4.** Effect of luteolin concentration on growth of *R. meliloti* 1021. A 5.0 μM luteolin treatment produced data nearly identical to the 2.5 μM values.

medium after being transferred from the yeast-extract maintenance medium.

Viable cell counts (cfu) and protein measurements of cultures growing with flavonoids indicated that those parameters increased with $A_{600}$ (*e.g.* after 21 h of growth, control cells had $A_{600} = 0.234$ with $1.1 \times 10^8$ cfu·mL$^{-1}$ and 66 μg protein·mL$^{-1}$, while cells treated with 5 μM quercetin had $A_{600} = 0.503$ with $3.5 \times 10^8$ cfu·mL$^{-1}$ and 134 μg protein·mL$^{-1}$). When cells were removed from the medium by centrifugation, $A_{600}$ values of the supernatant did not account for flavonoid effects (*e.g.* supernatant $A_{600}$ for both control and treated cells ranged from 0.007 to 0.015). Thus, extracellular polysaccharides were not responsible for the flavonoid-induced increases in optical density.

There was no detectable effect of carbon substrate on the luteolin increase in growth rate (Table II). Luteolin decreased the doubling time of *R. meliloti* 1021 cells with 0.25 to 10.0 g mannitol·L$^{-1}$ and with 1.0 g glucose·L$^{-1}$. There was no evidence that luteolin itself was metabolized in two experiments using 1.0 g mannitol·L$^{-1}$. When luteolin concentration was measured by HPLC in the minimal medium with *R. meliloti* 1021 cells, one-third of the luteolin disappeared in the first 24 h (*e.g.* 1.2 to 0.8 μM). However, data from flasks lacking bacteria showed that abiological losses, possibly adsorption to glass surfaces, occurred because those flasks, too, declined in luteolin concentration (*e.g.* 1.3 to 0.8 μM).

Chrysin (5,7-dihydroxyflavone), which has not been identified in alfalfa seed or root exudates, promoted growth rate of *R. meliloti* 1021 as much as luteolin or quercetin (Table III). Direct viable cell counts supported that conclusion: After 12 h of growth, $A_{600} = 0.082$ for control cells with $3.1 \times 10^7$ cfu·mL$^{-1}$, while cells exposed to 2.5 μM chrysin had $A_{600} = 0.207$ with $2.1 \times 10^8$ cfu·mL$^{-1}$. After removing bacterial cells by centrifugation, supernatants averaged $A_{600} = 0.007$. Neither 5- nor 7-hydroxyl substitutions alone affected *g* significantly, as measured by optical density. Cells exposed to apigenin had lower *g* values than those treated with luteolin, but that decrease was not significant in all experiments.

Luteolin and quercetin also affected growth of several other soil microorganisms, as measured by $A_{600}$ (Table IV). These flavonoids increased growth rate of all *R. meliloti* strains tested, including A2012, which is mutated in the three known copies of *nodD*. Growth rates of *R. trifolii* 102X99 and *P. putida* also were increased by luteolin and quercetin, but these flavonoids had no significant effect on growth of *A. tumefaciens* or *B. subtilis*. Growth of two *Pythium* species was slowed significantly by luteolin, quercetin, the methoxychalcone, and formononetin. Positive effects of luteolin and quercetin on growth rate of *R. meliloti* A2012, *R. trifolii*, and *P. putida*

**Table I.** *Effects of Alfalfa Flavonoids on Growth Rate (Doubling Time) of R. meliloti 1021*

Cells were inoculated into test media after one doubling in minimal control medium (1.0 g·L$^{-1}$ mannitol) following transfer from a medium containing yeast extract.

| Flavonoid[a] | Natural Source (Exudate) | Doubling Time[b] |
|---|---|---|
| | | *h* |
| None | | 11.5 b |
| Luteolin, 2.5 μM | Seed | 8.0 a |
| Luteolin-7-*O*-glucoside, 7.0 μM | Seed | 7.5 a |
| Quercetin, 1.0 μM | Seed | 7.5 a |
| Quercetin-3-*O*-galactoside, 8.0 μM | Seed | 7.8 a |
| 4,4′-Dihydroxy-2′-methoxychalcone, 8.0 μM | Root | 17.3 c |
| 4′,7-Dihydroxyflavone, 7.5 μM | Root | 15.0 c |
| 4′,7-Dihydroxyflavanone, 9.0 μM | Root | 15.3 c |
| Formononetin, 7.0 μM | Stressed Root | 17.1 c |

[a] Flavonoid concentrations were quantified by HPLC. [b] Values followed by dissimilar letters differ significantly ($P \leq 0.01$).

**Table II.** *Carbon Source Effects on Growth Promotion of R. meliloti 1021 by Luteolin*

Cells were transferred to experimental media after growing for several days in the minimal control medium containing 1.0 g·L$^{-1}$ mannitol.

| Carbon Source | Luteolin[a] | Doubling Time[b] |
|---|---|---|
| | | *h* |
| Mannitol, 0.25 g·L$^{-1}$ | None | 88.0 a |
| | 10 μM | 16.8 b |
| Mannitol, 1.0 g·L$^{-1}$ | None | 58.7 a |
| | 10 μM | 14.0 b |
| Mannitol, 10 g·L$^{-1}$ | None | 61.0 a |
| | 10 μM | 24.8 b |
| Glucose, 1.0 g·L$^{-1}$ | None | 40.6 a |
| | 10 μM | 21.2 b |

[a] Luteolin was prepared to 10 μM by gravimetric methods in a defined minimal medium. [b] Values followed by different letters indicate a significant luteolin effect ($P \leq 0.01$) within a single carbon treatment.

**Table III.** *Effects of Flavone Structure on Growth Rate in R. meliloti 1021*

Cells were transferred to experimental media after growing for several days in the minimal control medium containing 1.0 $g \cdot L^{-1}$ mannitol.

| Flavonoid[a] | Doubling Time[b] |
|---|---|
| | *h* |
| None | 49.2 c |
| Flavone | 86.7 c |
| 5-Hydroxyflavone | 58.8 c |
| 7-Hydroxyflavone | 83.4 c |
| 5,7-Dihydroxyflavone (chrysin) | 15.2 b |
| 4′,7-Dihydroxyflavone | 92.0 c |
| 3′,4′,7-Trihydroxyflavone | 37.7 c |
| 4′,5,7-Trihydroxyflavone (apigenin) | 10.7 a |
| 3′,4′,5,7,-Tetrahydroxyflavone (luteolin) | 17.7 b |
| 3,3′,4′,5,7,-Pentahydroxyflavone (quercetin) | 13.5 b |

[a] Flavonoids were prepared to 10 $\mu$M by gravimetric methods in a defined minimal medium. [b] Values followed by dissimilar letters differ significantly ($P \leq 0.01$).

measured by $A_{600}$ were confirmed by viable cell counts in separate tests. *R. meliloti* A2012, after 19 h of growth, had $A_{600} = 0.114$ for control cells with $3.8 \times 10^7$ cfu·$mL^{-1}$, while cells exposed to 2.5 $\mu$M quercetin had $A_{600} = 0.200$ with $5.6 \times 10^7$ cfu·$mL^{-1}$; for *R. trifolii* 102X99, 21 h of growth gave $A_{600} = 0.093$ for control cells with $2.7 \times 10^5$ cfu·$mL^{-1}$, while cells exposed to 2.5 $\mu$M luteolin had $A_{600} = 0.271$ with $5.8 \times 10^7$ cfu·$mL^{-1}$. *P. putida* C-58 growth on the minimal medium was quite different from that of the rhizobial strains tested. Soon after inoculation into minimal medium, cell numbers increased greatly without corresponding changes in $A_{600}$. Flavonoid effects, however, were evident. Thus, after 12 h of growth, $A_{600} = 0.044$ for control cells with $2.6 \times 10^6$ cfu·$mL^{-1}$, while cells exposed to 2.5 $\mu$M luteolin had $A_{600} = 0.066$ with $7.8 \times 10^6$ cfu·$mL^{-1}$.

## DISCUSSION

Results from this study supply the first clear data suggesting that plants control growth of soil microbes with ecochemical zones created by releasing structurally different flavonoids from seeds and roots. Whether flavonoid effects on chemotaxis in *R. meliloti* (4), also differ in those regions, can be tested now that distributions of natural flavonoids are known for alfalfa seed and root zones (Fig. 2; 17).

The effects of flavonoids on microbial growth are poorly understood. Legume seeds release compounds that inhibit rhizobial growth (25), and the flavonol myricetin was identified as one such molecule (6). Two isoflavonoids released naturally from soybean roots increased growth in one of two soybean rhizobial strains, but unexplained concentration effects made the results difficult to interpret (5). By identifying the dominant flavonoids released naturally from alfalfa seeds and roots and by testing their effects on rhizobial growth, the present work shows that flavonoids with hydroxyl substituents on the 5 and 7 positions (Fig. 1B), which is typical of those released from alfalfa seeds (*e.g.* luteolin and quercetin), increased growth rate of all *R. meliloti* strains tested on a defined minimal medium (Tables I, III, and IV). The promotive effect occurred in the low micromolar range with two carbon substrates, and a wide range of mannitol concentrations did not alter the luteolin effect (Fig. 4; Table II). In contrast, 5-deoxyflavonoids (flavone numbering system) from alfalfa root exudate (17, 18) did not promote growth of *R. meliloti* (Tables I and IV). Conclusions about growth based on $A_{600}$ values were supported by viable cell counts and protein values. Although a previous report showed that root exudate can

**Table IV.** *Effects of Alfalfa Flavonoids on Growth of Rhizosphere Microbes*

Doubling times for bacteria were measured in defined liquid media. Growth rate for fungi was determined on complex agar media. Bacteria were transferred to experimental media after growing for several days in minimal control medium containing 1.0 $g \cdot L^{-1}$ mannitol.

| Organism | Flavonoid[a] | | | | | |
|---|---|---|---|---|---|---|
| | None | L | Q | DHF | MCh | F |
| | *doubling time, h*[b] | | | | | |
| Bacteria | | | | | | |
| *A. tumefaciens* | 5.1 | 6.6 | 5.6 | 5.6 | 5.0 | 5.4 |
| *B. subtilis* | 3.4 | 3.4 | 3.5 | 3.6 | 3.6 | 3.6 |
| *P. putida* | 5.0 | 2.9 a | 3.2 a | 3.8 | 4.6 | 3.9 |
| *R. meliloti* 445 | 50.2 | 10.6 b | 14.6 b | 56.2 | 75.1 | 37.9 |
| *R. meliloti* 102F28 | 69.8 | 15.3 b | 22.6 b | 50.6 | 82.7 | 64.9 |
| *R. meliloti* A2012 | 37.9 | 9.8 b | 9.1 b | 36.0 | 44.9 | 37.2 |
| *R. trifolii* 102X99 | 64.4 | 11.6 b | 13.6 b | 60.4 | 51.4 | 46.7 |
| | *growth rate, $cm^2 \cdot h^{-1}$* | | | | | |
| Fungi | | | | | | |
| *P. irregulare* | 1.10 | 0.91 a | 1.03 a | 1.10 | 1.02 a | 1.05 a |
| *P. ultimum* | 1.07 | 0.84 a | 0.95 a | 1.04 | 0.90 a | 0.99 a |

[a] Flavonoids (L, luteolin; Q, quercetin; DHF, 4′,7-dihydroxyflavone; MCh, 4,4′-dihydroxy-2′-methoxychalcone; F, formononetin) were prepared to 10 $\mu$M by gravimetric methods. [b] Values followed by the letters a or b differ significantly from the flavonoid-free control at $P \leq 0.05$ or 0.01, respectively.

promote polysaccharide formation by rhizobia (3), that phenomenon did not have major effects on $A_{600}$ data in these experiments.

The flavonoid effect on growth probably does not involve rhizobial *nodD*. Genetic evidence for this conclusion is based on the fact that *R. meliloti* A2012, which is mutated in all *nodD* genes (12), still responds to luteolin and quercetin (Table IV). Other data show that, while quercetin increased growth rate in these experiments, it did not induce transcription of *nodC-lacZ* fusions controlled by $nodD_1$ (22) or $nodD_2$ (CA Maxwell, unpublished data).

Data reported here extend our knowledge of biologically active flavonoids that are released naturally by germinating alfalfa seeds. Quercetin-3-*O*-galactoside (Fig. 2, peak B) together with peak C, which releases quercetin on acid hydrolysis, account for 46% of the flavonoids in aqueous solution surrounding seeds after 4 h of imbibition. These results are consistent with analyses of 2-butanone extracts from "*Medicago* × *varia*" seeds (7) (apparently a hybrid of *M. sativa* L. × *M. falcata* L. [11]), which also identified quercetin-3-*O*-galactoside as a dominant flavonoid. The current evidence shows that this compound is released into aqueous environments where plant-microbe interactions occur. Luteolin conjugates and methoxylated derivatives comprise an additional 22% of the flavonoids released from these seeds, and the major form of that flavonoid, luteolin-7-*O*-glucoside (Fig. 2, peak A), also increases growth rates of *R. meliloti* (Table I; Fig. 3).

The complexities of bacterial growth are not fully understood (20), but plant flavonoids are not generally viewed as factors that promote the process. It is well known, of course, that isoflavonoid molecules often function as phytoalexins and inhibit microbial growth (24). The mechanism by which flavones with 5,7-dihydroxyl substitutions promote microbial growth is not apparent from data presently available. Without evidence that flavonoids substitute for a specific molecule, such as a vitamin, we choose to refer to them simply as growth promoters. The growth-promoting effects of luteolin and quercetin were observed only in a minimal medium that allows rather poor growth and not in the presence of yeast extract and tryptone. When rhizobial cells had been acclimated to minimal medium and showed very slow growth rates, the luteolin effect was quite large (Table IV). When *R. meliloti* cells had been exposed to minimal medium for a shorter period, growth rates of control cells were promoted less markedly by luteolin (Table I; Figs. 3 and 4). In this latter instance, factors remaining from previous exposure to yeast extract may have decreased the luteolin effect.

Luteolin and quercetin show some specificity in their capacity to increase growth of microorganisms that might be found in soil near germinating alfalfa seeds (Table IV). Growth of the benign bacterium *P. putida* was stimulated by these compounds, but the pathogen *A. tumefaciens* (27), which is taxonomically close to *Rhizobium*, was not affected. The fact that *R. trifolii* responded to luteolin and quercetin shows that growth promotion by a 5,7-substituted flavonoid can occur in other rhizobial organisms, but obviously luteolin or quercetin may not be the natural signals from other legumes. The inhibitory effects of several flavonoids on *Pythium* growth rates were significant and reproducible, but control mycelia were growing so rapidly that the 5 to 20% decreases in growth rate may not be biologically important.

Although one cannot extrapolate directly from these experiments to the soil environment, results reported here suggest that our current understanding of rhizosphere chemistry is rudimentary. Plants certainly affect primary metabolism of soil microbes through carbon released from their roots (28), but specific molecules within that carbon mass can have unique effects on microbial growth. Luteolin and quercetin in seed exudate may play a critical role in structuring the microbial community around the developing root during the first few hours of germination by promoting growth of the $N_2$-fixing alfalfa symbiont *R. meliloti*. Given the fact that increasing the number of rhizobial cells promotes root nodule formation (reviewed in ref. 1), the growth-enhancing effects reported here may have significant agricultural implications and could partially explain several previous reports (14, 26).

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